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Effect of Different Combinations of the Growth Factors and Hormones on *invitro* Maturation of Goat Preantral Follicles

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A B S T R A C T

A series of experiments were carried out with different combinations of various growth factors and hormones like Growth Hormone (GH), Insulin like Growth Factor-I (IGF-I), Epidermal Growth Factor (EGF), Thyroxin (T_4) and Follicle Stimulating Hormone (FSH) in culture medium to support growth and development of goat preantral follicles (PFs) (diameter ranging from 150-400µ) *in vitro* for a period of eight days and the retrieved oocytes from cultured preantral follicles were kept for incubation in *in vitro* maturation media (IVM) for 27 hours. The development of the cultured PFs was assessed by the proportions of follicles exhibiting growth, increase in follicular diameter, antrum formation and extrusion of oocytes *in vitro*. During eight days culture period, the proportion of PFs exhibiting antrum formation (89.33±1.86) and extrusion of oocyte (15.55±2.09) were highest when the medium was supplemented with T_4+FSH+GH+EGF (T_3) having a significant difference with follicles cultured in other treatments. Oocytes retrieved from PFs grown in different culture media were kept in IVM media. Oocytes obtained from T_3 treatment (T_4+FSH+GH+EGF) had maximum percentage of M-II stage oocytes (38.6%) when compared to the other treatments. From the present study, it can be concluded that, *in vitro* development of goat PFs could be significantly improved through addition of GH, EGF, T4 and FSH in best combinations.

Introduction

In mammals, the preantral follicles serve as store-house of majority of oocytes. The ovulatory follicle develops from primordial follicle by a process of folliculogenesis. Through this process, a healthy oocyte is usually selected for maturation. Recent advancements in the area of reproductive biotechnology, particularly the animal biotechnology is reaching to the level where it can be successfully utilized for augmenting the livestock productivity. Few current reproductive technologies used for increasing animal productivity are super ovulation, *in vitro* embryo production, nuclear transfer,

stem cell culture, transgenic animal production etc. Since these technologies depend on availability of large number of fertilizable oocytes, due to which need arises to produce large number of meiotically competent oocytes from *in vitro* culture of preantral follicles. Several *in vitro* studies suggest that the preantral follicles are able to generate competent oocytes that are able to undergo subsequent embryo development *in vitro* (Arunakumari *et al.,* 2010; Magalhães *et al.,* 2011b). Moreover, the follicular development is regulated by various endocrine and paracrine factors (Araújo *et al.,* 2011). The ovarian follicular development occurs due to action of various growth factors on the receptors that are expressed in a stage-specific form and act synchronically to promote stimulatory and/or inhibitory effects (Almeida *et al.,* 2011). Among various growth factors and hormones, Growth Hormone (GH), Insulin like Growth Factor-I (IGF-I), Epidermal Growth Factor (EGF), Thyroxin (T_4) and Follicle Stimulating Hormone (FSH) have been highlighted in the present study.

In several mammalian species different IVM culture systems have been established to study oocyte development, and have applied to assisted reproduction in humans and livestock animals. Mouse is the only species in which *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes from PFs resulted in the birth of live offspring (Eppig and O'Brien, 1996; Liu *et al.,* 2001). However among farm animals, success has been achieved up to embryo development using oocytes from cultured PFs of adult ovaries in pig (Wu *et al.,* 2001a and b), buffalo (Gupta *et al.,* 2008), sheep (Arunakumari *et al.,* 2010) and goat (Magalhaes *et al.,* 2011a) but none of these studies progressed to develop blastocyst except pig. Addition of GH (50ng/ml) resulted in growth of oocytes form goat preantral follicles that were acceptable for IVM and IVF Magalhaes *et al.,* (2011b). Addition of VEGF-A_{165} to the culture medium improved the development of goat PFs cultured *in vitro*, allowing the production of mature oocytes (Araujo *et al.,* 2011). The reported success in the meiotic maturation of sheep oocytes from cultured PFs has already been published by Arunakumari *et al.,* (2010) by using various growth factors and hormones. However, the study in goats is still awaited. So the present study was aimed to improve the meiotic maturation rate of oocytes by *in vitro* culture of goat PFs in different combinations/sequences of GH, IGF-I, EGF, T_4 and FSH.

Materials and Methods

Unless otherwise stated, all culture media, hormones, growth factors, PBS and chemicals were purchased from Sigma (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All media were incubated at 39^{o}C under a humidified atmosphere of 5% CO_2 in air for 2 h prior to use. Phosphate buffered saline (PBS), collection medium for PFs [HEPES buffered tissue culture medium 199 supplemented with 0.5% bovine serum albumin (BSA), 50 µg/mL gentamicin sulphate, 0.23 mM of sodium pyruvate, 2 mM l-Glutamine and 25 IU/mL heparin], handling medium (collection medium without heparin), stock solutions of EGF and FSH were all prepared as described by Tamilmani *et al.,* (2005). The preparations of thyroxin, GH and IGF-I solutions were made as per Arunakumari *et al.,* (2007, 2010). Bicarbonate buffered tissue culture medium 199 supplemented with 50 µg/mL gentamicin sulphate was used as control medium.

In vitro maturation media

Control media (TCM199B) was supplemented with 10 µg/ml FSH, 10 µg/ml LH, 1 µg/ml Estradiol 17β, 50 µg/ml gentamicin sulphate, 10µg/ml of Bovine serum albumin (FAF) and

10% fetal bovine serum (IVM-I). The prepared media was stored at 4°C for up to 1 week in 5 ml disposable syringe with 0.22 µm filter attached. Filtered medium was equilibrated for 1 h at 39 ^{0}C in 5% CO_2 in air under humidified atmosphere prior to use.

Preparation of propidium iodide solution

Stock solution of propidium iodide (PI) was prepared by reconstituting 5 mg of lyophilized desiccate of propidium iodide (P 81845, Sigma, USA) in 5 ml of filtered PBS to yield concentration of 1 mg/ml (1 µg/µl). 10 µl of this solution was diluted with 990 µl of filtered PBS to yield a final concentration of 10 µg/ml PI solution. This solution was stored in 1 ml of plastic aliquots at 4^{0}C in darkness till used.

Collection of ovaries, isolation, selection and culture of preantral follicles

Collection of ovaries, isolation of PFs, classification and selection of PFs for cultures were performed, as described by Arunakumari *et al.,* (2010) and Amin *et al.,* (2013). Briefly each ovary was cut into two halves along its longitudinal axis. The medulla is scooped out from each half. Then, each half of the ovarian cortex was dissected into thin slices using a 26 gauge needle and sterile surgical blade. Under a stereo zoom microscope (Nikon, Japan), these cortical slices were subjected to micro dissection in collection medium for isolation of the PFs, in the size range of 150-400 µm. The diameter of PFs was measured using Scopetek software, China. Care was taken to leave a small amount of stromal tissue attached to the basement membrane of the follicles. PFs having a centrally placed spherical oocyte with an intact basement membrane were chosen for culture. The selected PFs were washed thrice in handling and culture medium and placed individually in 20 µL droplet of culture medium in 35 mm tissue culture dishes. The micro droplets were overlaid with autoclaved pre-equilibrated mineral oil and cultured for 8 days in 5% CO_2 incubator at 39°C. Half of the culture medium was replaced with fresh culture medium every 48 h.

In vitro maturation of oocytes from in vitro cultured follicles

The *in vitro* cultured follicles were carefully opened (if the oocytes were not extruded itself) using two 26G needles attached to 1ml syringe barrels, to release the oocyte inside after the end of 8 days culture period. The collected oocytes were washed three times in holding medium followed by three washings in the *in vitro* maturation medium before being placed individually in ~20 µl droplets of IVM medium in 35-mm tissue culture dishes. The droplets were overlaid with autoclaved, pre-equilibrated lightweight mineral oil. These culture dishes were incubated at 39 °C in 5% CO_2 for 24 h in a CO_2 incubator. This procedure regularly supports meiotic maturation of more than 80% of oocytes collected from the antral follicles in sheep (Rao *et al.,* 2002).

Staining of *in vitro* matured oocytes

At the end of the IVM period of 27 hours, the oocytes were denuded of cumulus cells either by repeated pipetting through a fine-bore glass pipette or by placing COCs (Fig. A) in 200 µl of hyaluronidase solution for 1 minute and the cumulus cells were denuded off the oocytes by repeated pipetting through a fine bore glass pipette. The denuded oocytes were washed twice in filtered PBS to remove any hyaluronidase solution adhering to the oocytes. Denuded oocytes were placed separately 200 µl PI (Propidium Iodide) stain solution for 15 minutes in darkness in normal atmosphere. After the end of 15 minutes staining period, oocytes were washed twice in

filtered PBS to remove excess PI stain particles adhering to the oocytes.

Morphological evaluation of cultured preantral follicles and nuclear status of oocytes

Morphological evaluation of PFs was done as described by Amin *et al.*, (2013). However, the stained oocytes were examined through an inverted microscope at magnification of 400x with fluorescent illumination equipped with an excitation filter 510-530 nm, emition filter 590 nm and dichromatic mirror 570 nm and the nuclear maturation of oocytes was evaluated by observing the GV (Germinal Vesicle) (Fig. B), GVBD (Germinal Vesicle Breakdown) (Fig. C), M-II (Metaphase-II) (Fig. D), UC (Unclassified) stages of oocyte captured by using a digital camera coupled to microscope.

Statistical analysis

Two way ANOVA procedure, followed by Duncan's multiple range test in SPSS 17 was performed. Percentage data (proportions) was subjected to arcsine transformation prior to comparisons.

Results and Discussion

In the present study a total of eleven hundred (1125) preantral follicles were kept for *in vitro* culture in different treatments (T_1-T_5), out of these a total number of 931 oocytes were retrieved from preantral follicles of different treatments after 8 days of culture period, while remaining were damaged during culture and retrieval time. Among all treatments, T_3 were found to be the best combination as proportions of follicles exhibiting growth is maximum (98.00±0.87) which is significantly higher than control (T_5) ($P \leq 0.05$), however, it does not vary significantly with other treatments (T_1, T_2 and T_4). This treatment combination also showed better results with respect to average increase in diameter (42.66±2.06), antrum formation (89.33±1.86) and extrusion of oocytes (15.55±2.09) than T_1, T_2, T_4 and T_5 (Table 1). Interestingly, none of the oocytes got extruded from control medium. Oocytes, having at least two layers of cumulus cells and evenly granular cytoplasm, retrieved from different treatments of preantral follicular culture after a period of eight days were kept for *in vitro* maturation in incubator at 38.5^{o}C, 5% CO_2 in IVM media for 27 hours (Table 2). The oocytes collected by aspiration method from antral follicles of ovaries were kept for *in vitro* maturation as control-II. In order to evaluate the nuclear status of oocytes, surrounding cumulus cells were denuded off and stained with propidium iodide (PI).

The nuclear status was determined by marking the changes like exhibition of metaphase II stage, Germinal vesicle breakdown (GVBD), shedding of polar body after putting oocytes in IVM media. From the present results (Table 2) it can be confirmed that treatment T_3 showed higher rate of nuclear maturation as compared to other treatments T_1, T_2, T_4, T_5 and T_6. Oocytes from T_3 in the IVM medium did not differ significantly with T_1, T_2, T_4, T_5 and T_6 with respect to parameters like GV and GVBD. However, the maturation rate in the control medium was significantly lower ($P \leq 0.05$) than other treatment groups (Table 2). The present study was further proceeded to perform the comparative study on meiotically competent oocyte (M-II stage) production, after *in vitro* maturation in IVM media for oocytes obtained from PFs cultured for a period of eight days in different treatments.

In the present study, there was no significant difference observed for oocytes exhibiting GVBD, GV stage of nuclear status in the treatment T_1, T_2, T3, T_4 and T_6 of IVM media. However, none of the oocyte retrieved from PFs cultured in control (T_5) were matured to GVBD stage.

Table.1 Effect of various growth factors and hormones in different combinations on *in vitro* development of goat preantral follicles

Treatments	Combinations of different hormones (Replicates/ No. of follicles)	Proportion of PFs exhibiting growth (Mean ± SE)	Average increase in diameter (µ) of PFs (Mean ± SE)	Proportion of PFs exhibiting antrum formation (Mean ± SE)	Proportion of PFs ovulated (Mean ± SE)
T_1	T_4 + FSH (45/225)	95.55±1.25^b	29.51±0.81^b	74.22±1.75^b	1.33±0.75^a
T_2	T_4 + FSH + EGF (45/225)	97.77±0.94^b	34.62±0.71^c	80.44±1.85^c	5.77±1.36^b
T_3	T_4 + FSH + GH +EGF (45/225)	98.00±0.87^b	42.66±2.06^d	89.33±1.86^d	15.55±2.09^d
T_4	T_4 + FSH+IGF +EGF (45/225)	97.33±1.02^b	41.00±1.33^d	82.22±1.58^c	11.11±2.34^c
T_5 (Control)	Control Media (45/225)	40.00±1.68^a	13.73±0.58^a	29.33±1.96^a	0.00^a

Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Table.2 Nuclear status and MII stage oocytes production of oocytes obtained from cultured PFs after 27hrs *in vitro* maturation in IVM media

Treatments	Combinations (Replicates/ No. of oocytes)	GV (%)	GVBD (%)	M-II (%)	Un Classified (%)	MII stage oocytes production
T_1	T4 +FSH (6/48)	21.38±2.21^a	34.44± 3.8^b	19.07±2.17^b	25.09±3.71^c	19.07±2.17^b
T_2	T4+FSH+EGF (6/41)	17.07±1.44^a	31.68±4.21^b	26.12±2.81^{bc}	20.88 ± 4.56^{bc}	26.12±2.81^{bc}
T_3	T4+FSH+GH+EGF (6/46)	26.97±2.24^a	26.03±2.77^b	38.06±2.93^d	10.73±2.33^{ab}	38.06±2.93^d
T_4	T4+FSH+IGF+EGF (6/46)	24.24±2.96^a	34.81±3.84^b	29.61 ±4.11^c	11.28±3.80^{ab}	29.61 ±4.11^c
T_5 (Control-I)	Control-I (6/40)	55.64±2.81^b	0.00^a	0.00^a	44.35±2.81^d	0^a
T_6 (Control-II)	Control-II (6/75)	17.17±1.97^a	32.62±3.19^b	45.74±1.70^e	4.44±2.04^a	45.74±1.70^e

Values with different superscripts within a column are significantly different ($P \leq 0.05$).

Statistically significant number of oocytes retrieved from PFs cultured in control media (T_5) were in GV stage (55.64 ±2.81) compared to the treatments T_1, T_2,T_3, T_4 and control-II (T_6). Highest ($P \leq 0.05$) numbers of oocytes from control-II (antral oocytes, T6) were matured to MII stage (45.74±1.70). However, none of the oocyte retrieved from PFs cultured in control (T_5) were matured to MII stage. Among the treatments, oocytes

retrieved from PFs cultured in T_3 had exhibited the highest ($P \leq 0.05$) percentage of nuclear maturation to MII stage (38.06±2.93) when compared to PFs of other treatments T_1, T_2 and T_4.

The present study demonstrated the effect of various growth factors and hormones like IGF, EGF, T_4, FSH and GH in different combinations on *in vitro* development of goat preantral follicles. The present study advocated that the highest proportion of PFs exhibiting growth, increase in diameter, antrum formation and *in vitro* ovulation were in treatment groups cultured in combination of hormones and growth factors T_4+FSH+GH+EGF (T_3). These results are comparable with the findings observed by Zhou and Zhang (2005) who reported that, in presence of FSH, IGF-I in combination with EGF produced a higher survival rate of adult caprine preantral follicles than any other growth factor did individually. Findings of present study are in accordance with findings of Magalhaes *et al.,* (2011b) in high rate of follicular survival and antrum formation when media supplemented with Growth hormone (GH). Furthermore, the influence of the thyroxin on PFs appears to depend on the physiological status of the PFs at the time of collection (Gupta *et al.,* 2007). Hence the present study proved that the *in vitro* development of goat PFs could be significantly improved through addition of T_4, FSH, GH, EGF, and IGF-I in different combinations in the culture medium. Moreover, according to Gupta *et al.,* (2007) age of the animal and season of the year at the time of isolation will also influence the *in vitro* development of preantral follicles.

However, immature oocytes *in vivo* remain arrested in the first meiotic prophase until maturation is induced by the interaction of steroids, gonadotropins and other follicular constituents. Full oocyte maturation involves not only acquisition of meiotic competency, but also cytoplasmic maturation. Use of different culture and maturation media *in vitro* with different supplements might improve the understanding of the minimal requirements for oocyte growth and maturation, expected to provide a reasonable yield of meiotically competent oocytes. Oocytes retrieved from PFs were grown in IVM culture media. In IVM media oocytes obtained from T_3 treatment (T_4+FSH+GH+EGF) had maximum percentage of M-II stage oocytes (38.6%) in contrast to the findings of Arunakumari *et al.,* (2010) in sheep, the difference might be due to variation in culture media or species difference. However, these findings were in accordance with the Magalhaes *et al.,* (2011b) on production of matured oocytes by supplementation of Growth hormone at a concentration of 10 ng/ml and 50 ng/ml. The present findings were in accordance with the results of Saraiva *et al.,* (2012) who studied the effect of both FSH and LH on oocyte maturation. Meiotic resumption of oocytes from the present study was similar to the observations made by Saraiva *et al.,* (2012) with the media supplemented with LH at concentration 50 ng/ml. However, difference was noticed with LH supplemented at 100 ng/ml. It was reported by earlier studies that the effect of LH on the *in vitro* culture of preantral follicles depends on the follicular category, the concentration used and the timing of addition of LH to the culture medium (Tamilmani *et al.,* 2005; Silva *et al.,* 2011).

Most IVM protocols do employ luteinizing hormone (LH) or follicle stimulating hormone (FSH) or estradiol or a combination of them. Saraiva *et al.,* (2012) reported that FSH increases the levels of LH receptor mRNA during *in vitro* culture of secondary follicles, ensuring a synergistic effect of both gonadotropins on follicular growth and

meiotic resumption of oocytes recovered from goat preantral follicles cultured *in vitro.*

Chaves *et al.*, (2012) reported that medium supplemented with FSH and Insulin at 10 ng/ml presented oocytes, with higher rates of meiosis resumption as well as oocytes in metaphase II. The effect of both FSH and LH combination were studied earlier by Saraiva *et al.*, (2012), the present findings were in accordance with the results of Saraiva *et al.*, (2012).

From the present study, it can be concluded that, in *vitro* development of goat PFs could be significantly improved through addition of GH, EGF, T_4 and FSH in best combinations.

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